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Biochemical Oxidation of D-Sorbitol to L-Sorbose by Immobilized *Gluconobacter Oxydans* Cells

Scientific Note

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INTRODUCTION

Immobilized cell biocatalysts have been extensively investigated in the past 10 years (*1*). Utilizing such biocatalysts allows application of continuous and stationary production modes, convenient technology control, higher product purity, and so on (*1,2*).

Microbial oxidation of D-sorbitol to L-sorbose is an important part of vitamin C production. It is performed in batch reactors by free *Gluconobacter oxydans* (Gl. Ox.) cells.

Immobilized *Gl. Ox.* cells have been investigated by several authors (*3–6*). The most popular approach to immobilize whole cell is entrapment in the microscopic network of natural and synthetic gels. Entrapment is easily done, and enables high retention of cell viability. A disadvantage of these biocatalysts is the high diffusion barrier caused by the microscopic gel network. Oxidizing activity of *Gl. Ox.* cells entrapped in Ca-alginate was investigated by oxidizing glucose to gluconic acid at high glucose

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concentrations (*3*), glycerol to dihydroxyacetone (*4,5*), and D-sorbitol to L-sorbose (*6*).

Because of low Ca-alginate gel stability, various stabilization methods have been applied to extend biocatalysts durability, such as (1) polyethyleneimine treatment followed by glutaraldehyde, and (2) *N*-hydroxysuccinimide treatment followed by polyethyleneimine (*7*). The least toxic and most easily done is Al^{3+} treatment (*8*). There are also some references for *Gl. Ox.* cells immobilized in polyacrylamide gel (*9–11*).

A suitable method for entraping viable cells is proposed by Freeman and Aharonowitz (*12*). The gel is made of dialdehyde crosslinked polyacrylamide partially substituted with acylhydrazide groups. It has good mechanical, chemical, and biological stability and the entrapped cells retain a high activity. To prepare bead-shaped biocatalysts, the method (*13*) may be modified by adding small amounts of Na-alginate to the polyacrylamidehydrazide solution.

With this work, we intended to prepare biocatalysts for converting D-sorbitol to L-sorbose using immobilized *Gl. Ox.* cells with good mechanical and chemical stability, high oxidation activity, and low diffusion limitations.

METHODS

Microorganisms

We used *Gl. Ox.* NBIMCC 902 strain, stored on sorbitol agar at 4°C.

Chemicals

Na-alginate and glutaraldehyde (25% water solution) were obtained from Fluka AG, Switzerland; yeast extract and $CaCl_2$ were obtained from Serva, FRG; glyoxal hydrate and $Al(NO_3)_3$ were obtained from Merck, FRG; polyacrylamidehydrazide (PAAH) with a acylhydrazide group content of 0.15 mmol/g and 1×10^5 MW, and oxidized polyvinylalcohol (OPVA) with an aldehyde group content of 4.50 mmol/g and 2×10^3 MW were synthesized as described in (*12*).

Immobilization

Gl. Ox. cells were immobilized in several gels prepared as follows (Note: All solutions use water as the solvent.):

Method 1

A 1 mL suspension of *Gl. Ox.* cells (19×10^7 cells/mL) were mixed with 5 mL of sterile 30, 60, or 80 g/L Na-alginate. This suspension was added dropwise to freshly prepared, sterile 0.2, 0.4, or 0.6 *M* $CaCl_2$. The beads thus formed were left 2 h at 4°C and washed thoroughly with sterile distilled water.

Method 2

The gel beads prepared in Method 1 were put in 0.1 *M* $Al(NO_3)_3$ for 5 min and washed with sterile distilled water.

Method 3

Four milliliters of sterile PAAH (22.5 g/L) and Na-alginate (7.5 g/L) were mixed with a 1 mL cell suspension (19×10^7 cells/mL) and 0.2 mL of 100 g/L OPVA. This suspension was quickly added dropwise to cool (4°C) crosslinking solution (0.4 *M* $CaCl_2$, 0.02 *M* glutaraldehyde). After 2 h, the beads were washed thoroughly with sterile distilled water and were ready for use.

Method 4

The cell suspension in PAAH (22.5 g/L) and Na-alginate (7.5 g/L) was prepared as in Method 3, but without OPVA. It was added dropwise to cool (4°C) crosslinking solution (0.4 *M* $CaCl_2$, 0.02 *M* glyoxal). Then the beads were treated as in Method 3.

Cultivation

The oxidation of D-sorbitol to L-sorbose by immobilization of *Gl. Ox.* cells was performed in 500 mL Erlenmeyer flasks shaken at 220 rpm and 30°C. The cultivation medium contained 100, 150, or 200 g/L D-sorbitol and 5 g/L yeast extract. After the complete conversion of D-sorbitol to L-sorbose, the beads were washed with sterile distilled water and were ready for use in the next cycle.

Analyses

The L-sorbose concentration in the cultivation medium was determined by the method by Schoorl (*14*). The analysis was carried out periodically several times each cycle.

Mechanical strength of beads was characterized by the crushing strength measured with a "Zwick" 1474 dynamometer (Ulm, FRG). Each reported value was an average of 10 measurements. The bead diameter was 2.50 ± 0.20 mm.

RESULTS

Figure 1 shows the impact of Na-alginate concentration on the oxidizing activity. The oxidizing activity was measured as the mean velocity of D-sorbitol transformation to L-sorbose (in g/L/h). The number of cycles without visible bead disintegration was adopted as a measure for biocatalyst stability. The Na-alginate beads survived five to eight cycles.

To extend the biocatalysts durability, it was treated with Al^{3+}. Figure 1 shows the Al^{3+} increased stability to 12 cycles while also having a slight benefit on oxidation activity.

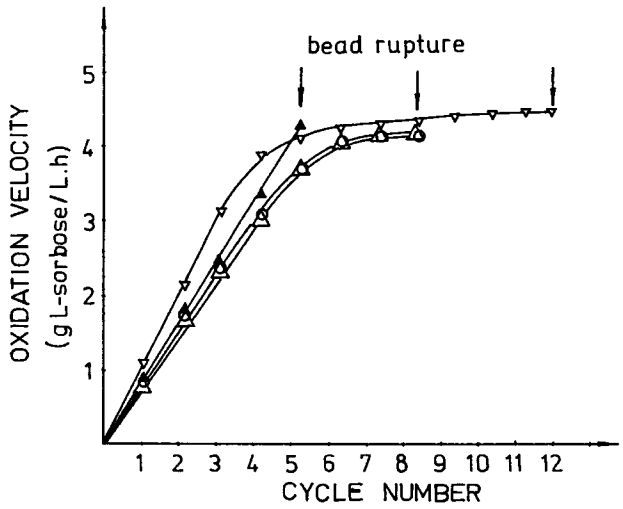

Fig. 1. Oxidation activity of beads prepared from solutions with different initial Na-alginate concentrations: 30 g/L ▲——▲ (disintegrated after the fifth cycle), 60 g/L ○——○ (disintegrated after the eighth cycle), 80 g/L ▲——▲ (disintegrated after the eighth cycle), 60 g/L stabilized with Al^{3+} ▽——▽ (disintegrated after the twelfth cycle).

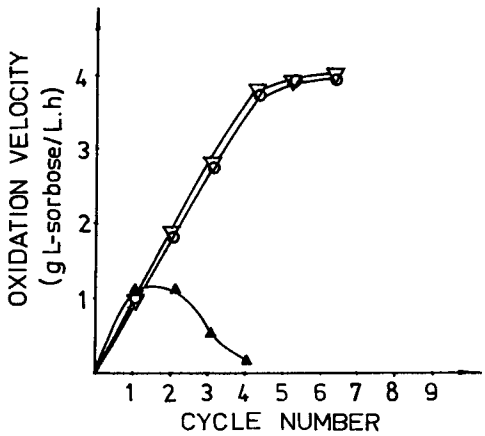

Fig. 2. Oxidation activity of biocatalysts crosslinked with different $CaCl_2$ concentrations: 0.2 *M* ○——○, 0.4 *M* ▽——▽, 0.6 *M* ▲——▲ (Note: Initial Na-alginate concentration was 60 g/L).

It has been shown (*15*) that increasing the Ca^{2+} concentration decreases the oxidizing activity of immobilized biocatalysts in Ca-alginate. Our results confirm this (*see* Fig. 2).

Figure 3 shows the oxidation activity level of *Gl. Ox.* cells immobilized in PAAH–Na-alginate crosslinked with OPVA–glutaraldehyde–Ca^{2+} (Method 4). The D-sorbitol concentration was 100 g/L, the biocatalyst content in the cultivation medium was 150 g/L, and the initial pH was 5.7. The biocatalysts prepared by Methods 3 and 4 have equal oxidation veloc-

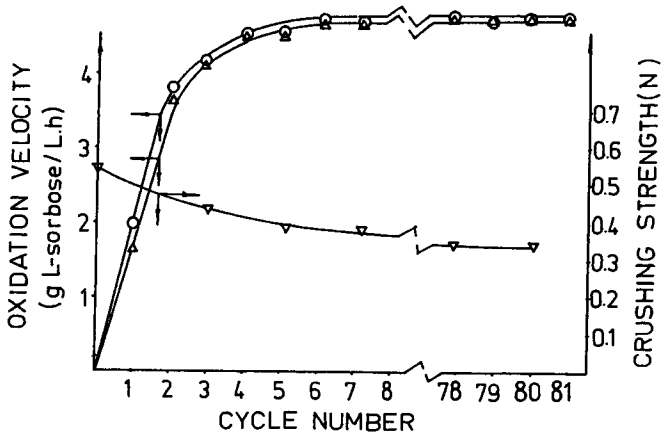

Fig. 3. Oxidation activity and the mechanical strength of biocatalysts prepared by crosslinking PAAH-Na-alginate: O——O oxidation activity of beads prepared by Method 3, △——△ oxidation activity of beads prepared by Method 4, ▽——▽ mechanical strength of beads prepared by Method 4.

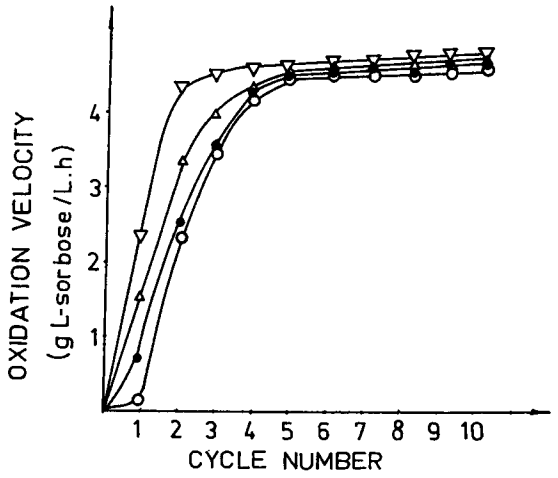

Fig. 4. Oxidation activity of biocatalysts prepared with different cell suspension concentrations by Method 4: O——O 8×10^4 cells/mL, ●——● 7×10^6 cells/mL, △——△ 5×10^8 cells/mL, and ▽——▽ 1×10^9 cells/mL.

ity of 4.5 g L-sorbose/L/h after the third cycle. The high oxidizing activity have been retained for more than 80 cycles (one cycle is about 22 h). Figure 3 shows bead crushing strength reduced slightly with increased cycle number.

The high stability and oxidation activity of PAAH-Na-alginate biocatalysts justified further investigations in immobilization and oxidation conditions.

Figure 4 shows the influence of initial cell suspension concentration (8×10^4, 7×10^6, 5×10^8, and 1×10^9 cells/mL) on the oxidation activity. Immobilization was performed as described in Method 4. The cultivation

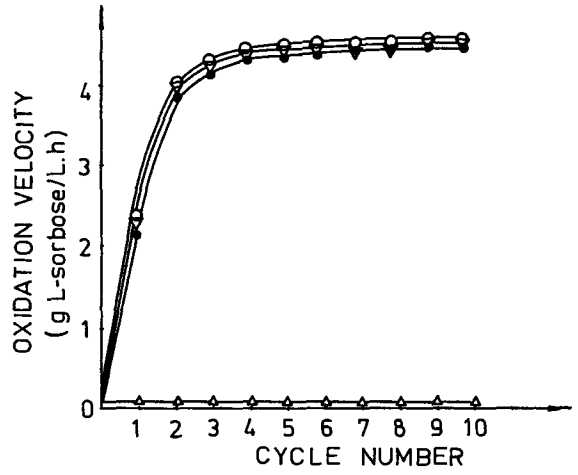

Fig. 5. Oxidation activity of biocatalysts prepared by Method 4 in cultivation medium with different initial pH: △——△ pH 3.0, ○——○ pH 4.5, ●——● pH 6.0, ▽——▽ pH 7.5.

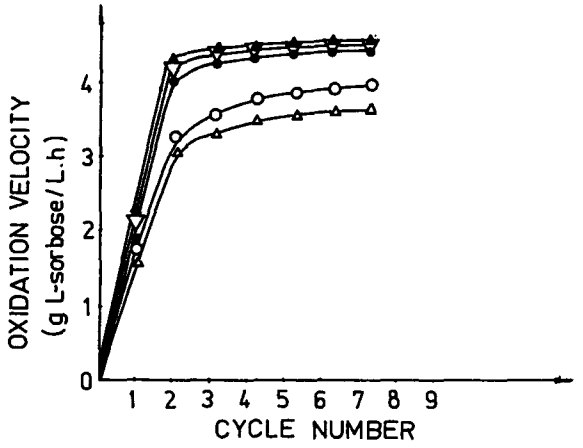

Fig. 6. Oxidation activity of biocatalysts prepared by Method 4 using different biocatalyst contents in the cultivation medium: 30 g/L △——△, 60 g/L ○——○, 150 g/L ●——●, 300 g/L ▽——▽, and 450 g/L ▲——▲.

conditions were the same as presented in Fig. 3. The lower cell concentrations initially had a lower activity until the intraparticle cell concentrations built up.

The influence of the cultivation medium initial pH on the oxidation activity of cells immobilized in crosslinked PAAH-Na-alginate (Method 4) is shown in Fig. 5. The D-sorbitol concentration was 100 g/L and the biocatalyst content was 150 g/L. There have not been observed any oxidation at pH 3. In the pH range 4.5–7.5, the oxidation proceeds with equal oxidation velocity (4.5 g of L-sorbose/L/h after the third cycle).

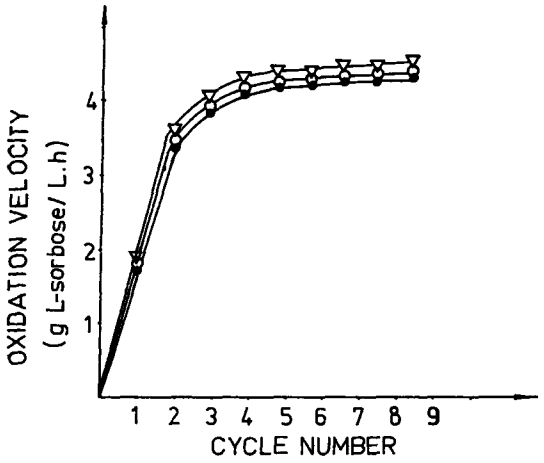

Fig. 7. Oxidation activity of biocatalysts prepared by Method 4 in different substrate concentrations: ●——● 100 g/L, ▽——▽ 150 g/L, and ○——○ 200 g/L.

Figure 6 shows the impact of biocatalyst content on oxidizing activity. The biocatalyst was prepared as described in Method 4. The D-sorbitol concentration was 100 g and the initial pH was 5.7. The biocatalyst content in the cultivation medium was 30, 60, 150, 300, and 450 g/L. The oxidation velocity (4.2 g of L-sorbose/L/h) was constant at the biocatalyst content over 150 g/L.

The influence of substrate concentration on the oxidizing activity of cells immobilized in crosslinked PAAH–Na-alginate (Method 4) is shown in Fig. 7. The biocatalyst content was 150 g/L and the initial pH was 5.7. The oxidation velocity after the third cycle stays constant (4.2 g of L-sorbose/L/h) in the range of 100–200 g/L D-sorbitol.

DISCUSSION

The immobilization of *Gl. Ox.* in Ca-alginate has the advantage of retaining high cell activity. The gel micronetwork has pore size of 50–1500 Å (*16*) and does not present a diffusion barrier for low molecular substrates and products. A great disadvantage of this biocatalyst is the low stability in the cultivation medium. In our experiment, we significantly extended the stability. Figure 1 shows that gels prepared from solutions with different concentrations of Na-alginate have different stabilities. The gel prepared with 30 g/L initial concentration is unstable, and disintegrates after the fifth cycle. The stability of gels prepared from 60 and 80 g/L of Na-alginate (initial concentrations) was equal (eight cycles before disintegration), but there is disadvantage working with 80 g/L solution because of its high viscosity. We consider a 60 g/L solution as the optimal concentration of Na-alginate for immobilizing *Gl. Ox.*

Extending biocatalyst durability by increasing the crosslinked agent (Ca^{2+}) concentration is limited to a 0.4 *M* solution of $CaCl_2$ because cell oxidation activity is inhibited (*see* Fig. 2). The most effective treatment was Na-alginate gel stabilized with Al^{3+} ions (Fig. 1), since it was stable for 12 cycles. The crosslinked three-dimensional structure was more stable than the one prepared solely with Ca^{2+} ions.

Even Al^{3+}-stabilized biocatalysts are not stable enough in cultivation medium. However, the stability is sufficient with biocatalysts immobilized in PAAH–Na-alginate crosslinked with OPVA–glutaraldehyde–$CaCl_2$. In addition, the cells immobilized by this method have a high, constant oxidation activity equal to the free cells, as observed for more than 80 cycles (Fig. 3). The high oxidation activity results from the low toxicity of the reagents (glyoxal, glutaraldehyde). In the mild conditions employed, the dialdehydes react rapidly and selectively with PAAH–acylhydrazide groups rather than the amino groups in cell proteins (*12*). In addition, their three-dimensional network does not hinder diffusion of D-sorbitol and L-sorbose because the biocatalyst pore sizes are 10 times greater than that of Ca-alginate (*17*). The open structure allows part of the proliferating *Gl. Ox.* cells to leave the gel and take part in oxidation as free cells. The high biocatalyst stability is caused by the strong network resulting from the crosslinking of PAAH with dialdehydes. The mutual intertangling of the two emerging PAAH–dialdehyde and Ca-alginate networks is further stabilized by hydrogen bonds emerging between the macromolecules. The biocatalyst is mechanically stabile since the beads crushing strength is almost unchanged with the time (*see* Fig. 3).

We investigated the influence of the initial cell suspension concentration on the oxidation activity (Fig. 4) and found that it differs in the first cycles only. After the first cycles, the proliferating cells fill up the gel beads and enter a stationary phase of oxidation activity.

The highest oxidation was established during the stationary phase of beads prepared with the highest cell concentration (1×10^9 cells/mL).

The influence of cultivation pH on the oxidation activity (Fig. 5) showed that low pH (about 3.0) inactivates the biocatalyst, whereas high oxidation activity is retained in the broad pH range of 4.5–7.5.

The impact of biocatalyst content on the oxidation activity was examined in the 30–450 g/L range (*see* Fig. 6). We established that raising the biocatalyst concentration above 150 g/L did not increase the oxidation activity since we reached "substrate saturation" of the biocatalyst. Exceeding that concentration led to low effectiveness.

D-sorbitol concentrations did not influence the oxidation activity in the range of 100–200 g/L of D-sorbitol, and the oxidation proceeded with almost the same velocity (*see* Fig. 7).

There is no difference in the oxidation activity and stability between the PAAH–Na-alginate biocatalysts joined by the two types of crosslinkers. Immobilization with glyoxal–$CaCl_2$ crosslinking is a little simpler and was preferred to OPVA–glutaraldehyde–$CaCl_2$.

CONCLUSION

We showed that immobilizing *Gl. Ox.* cells in crosslinked PAAH–Na-alginate showed high and constant oxidation of D-sorbitol to L-sorbose (comparable with that of the free cells) for a long period of time (more than 80 cycles).

REFERENCES

1. Mattiasson, B., ed. (1983), *Immobilized Cells and Organels,* vols. 1 and 2, CRC, Boca Raton, FL.
2. Linko, P. and Linko, Y. (1984), *CRC Rev. Biotechnol.* **1,** 289.
3. Tramper, J., Luyben, M., and van den Tweel, M. I. (1983), *Appl. Microbiol. Biotechnol.* **17,** 13.
4. Holst, O., Enfors, S., and Mattiasson, B. (1982), *Appl. Microb. Biotechnol.* **14,** 64.
5. Aldercrentz, P., Holst O., and Mattiasson, B. (1985), *Appl. Microbiol. Biotechnol.* **22,** 1.
6. Japanese Patent No. 110674 (1982).
7. Birnbaum, S., Pendleton, R., Larsson, P., and Mosbach, K. (1981), *Biotechnol. Lett.* **3,** 393.
8. Rochefont, W., Rehg, T., and Chan, P. (1986), *Biotechnol. Lett.* **8,** 115.
9. Pomortseva, N. V. and Krassilnikova, T. M. (1983), *Khim. Pharm.* **17,** 721.
10. Schnarr, G. W., Szarek, W. A., and Jones, I. K. N. (1977), *Appl. Environ. Microbiol.* **33,** 732.
11. Stefanova, S., Koseva, M., Tepavitcharova, I., and Beshkov, V. (1987), *Biotechnol. Lett.* **7,** 475.
12. Freeman, A. and Aharonowitz, Y. (1981), *Biotechnol. Bioeng.* **23,** 2747.
13. Bettmann, H. and Rehm, H. (1984), *Appl. Microbiol. Biotechnol.* **20,** 285.
14. Schoorl, N. L. (1929), *Untersuch Lebensmittel* **57,** 566.
15. Hertmier, W. and Heinrichs, A. (1986), *Biotechnol. Lett.* **8,** 567.
16. Andersen, I. L., Skipnes, O., Smidsrod, O., Ostgaard, K., and Hemmer, P. C. (1977), *ACS Symp. Ser.* **48,** 361.
17. Freeman, A. (1984), *Ann. N.Y. Acad. Sci.* **434,** 418.